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●*Original Contribution*

INFLUENCE OF TISSUE PRESERVATION METHODS ON ARTERIAL GEOMETRY AND ECHOGENICITY

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Abstract—Thoracic porcine aortas from 5 pigs were investigated with 7.5-MHz ultrasound *in vitro* at low and high transmural pressure before and after the following tissue preservation methods were applied: 1. Storage in frozen condition (−12°C) for 24 h followed by thawing; 2. fixation in formalin at zero transmural pressure for 24 h; and 3. fixation with formalin for 24 h while applying 74 mmHg of transmural pressure from within the lumen and a tensile force to longitudinally stretch the artery. Fixation in formalin at zero transmural pressure resulted in swelling of the arterial wall ($25 \pm 40\%$, $p < 0.02$, at low transmural measurement pressure) and in decreased echogenicity ($-23 \pm 38\%$, $p < 0.01$) of the arterial vessel wall. No changes in this respect were found after storage in a frozen condition nor after fixation in formalin at high transmural pressure which, therefore, are more appropriate procedures for fixation of arteries prior to *in vitro* ultrasound examination if geometry is important. © 1997 World Federation for Ultrasound in Medicine & Biology.

Key Words: **Ultrasound, Ultrasonic measurement, Vessel diameter, Wall thickness, Measurement of echogenicity, Freezing of specimen, Formalin fixation, Pressurized formalin fixation, Porcine artery.**

INTRODUCTION

Ultrasound investigation of human arteries has gained widespread acceptance in many clinical situations and, in some cases, treatment is based on ultrasound examination alone. A predominant example is examination of the carotid arteries, which has been developed over more than three decades. Atherosclerotic deposits (plaque) on the internal carotid artery may cause stroke, and surgical removal of the plaque has proven advantageous in the attempt to reduce the risk of stroke (ECST 1991; NASCET 1991).

To date, the main effort has been directed towards assessing the severity of the atherosclerotic narrowing in terms of hemodynamic importance. Recent research (Geroulakos et al. 1994; Langsfield et al. 1989) indicates that the appearance of the carotid plaque on ultrasound B-mode images may be of importance in relation to pathology. Specifically, echopoor plaques with a heterogeneous appearance may be among those most likely to cause a stroke (Cave et al. 1995; Geroulakos et al. 1994; Langsfield et al. 1989). Using intravascular ultrasound, Honey et al. (1992) have shown that soft (echopoor) plaques in the coronary arteries are less likely to fracture after balloon angioplasty and the treatment is, therefore, not durable due to elastic recoil (*i.e.*, the plaque expands back into the lumen after the balloon is removed). Thus, geometry and ultrasound reflectivity are parameters that may provide important information for diagnosis and treatment of vascular diseases.

To investigate the correlation between ultrasound images and pathological findings, *in vitro* studies of excised arteries are commonly carried out. Freshly removed arteries may be examined by ultrasound without any preparation but, to keep the specimen for more than 24 h and to perform histological analysis, some method of tissue preservation is necessary. The most widespread method is fixation in formalin. However, to extrapolate from *in vitro* to *in vivo* findings, knowledge of the effect of fixation on geometry, functionality and ultrasound echogenicity is crucial. The influence of different methods of tissue preservation has been investigated for a number of different tissues (Bamber et al. 1979; Crosby and Mackay 1978; Kremkau et al.

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1981; Park et al. 1993; Siegel et al. 1985; van der Steen et al. 1991) but, to our knowledge, investigations regarding vascular tissues have been only scarcely reported (Park et al. 1993; Siegel et al. 1985).

The purpose of the present study was to investigate the influence of the following 3 methods of tissue preservation on vessel geometry and ultrasound reflectivity:

- Freezing at −12°C for 24 h with subsequent thawing of the artery.
- Fixation in formalin at 21°C for 24 h at zero transmural pressure.
- Fixation in formalin at 21°C for 24 h while applying 74 mmHg of transmural pressure from within the lumen of the vessels and applying a tensile force along the length of the artery to stretch it longitudinally to match the *in vivo* length as closely as possible.

Thus, the first method has the potential of preserving vessel geometry and elasticity while the second and third methods render the artery rigid in a relaxed state and in a state matching a given blood pressure in the *in vivo* situation, respectively.

MATERIALS AND METHODS

Arterial samples and experimental setup

The thoracic aortas (elastic artery) from 5 pigs (weight 16–46 kg) were excised immediately after death. Branches were either ligated or sutured. As illustrated in Fig. 1a, each artery was subsequently cut into 3 segments corresponding to the 3 different tissue preservation methods described later. Each end of an artery segment was fixed to a specially designed acrylic fitting. Each fitting had the form of a 15-mm-long tube with a thread in one end for connection to an extension tube. The other end was smooth, but contained a notch at the tip for (waterproof) connection of the artery segment, as illustrated in Fig. 2. The connection between the fitting and the tube was waterproofed by a rubber O-ring. The fittings remained on the artery segments during the entire experiment. The fittings were marked with artery segment identification numbers, which also indicated the position in the vessel holder. The use of experimental animals was in accordance with the guidelines set forth by the Danish Ministry of Justice.

The artery segment with fittings and extension

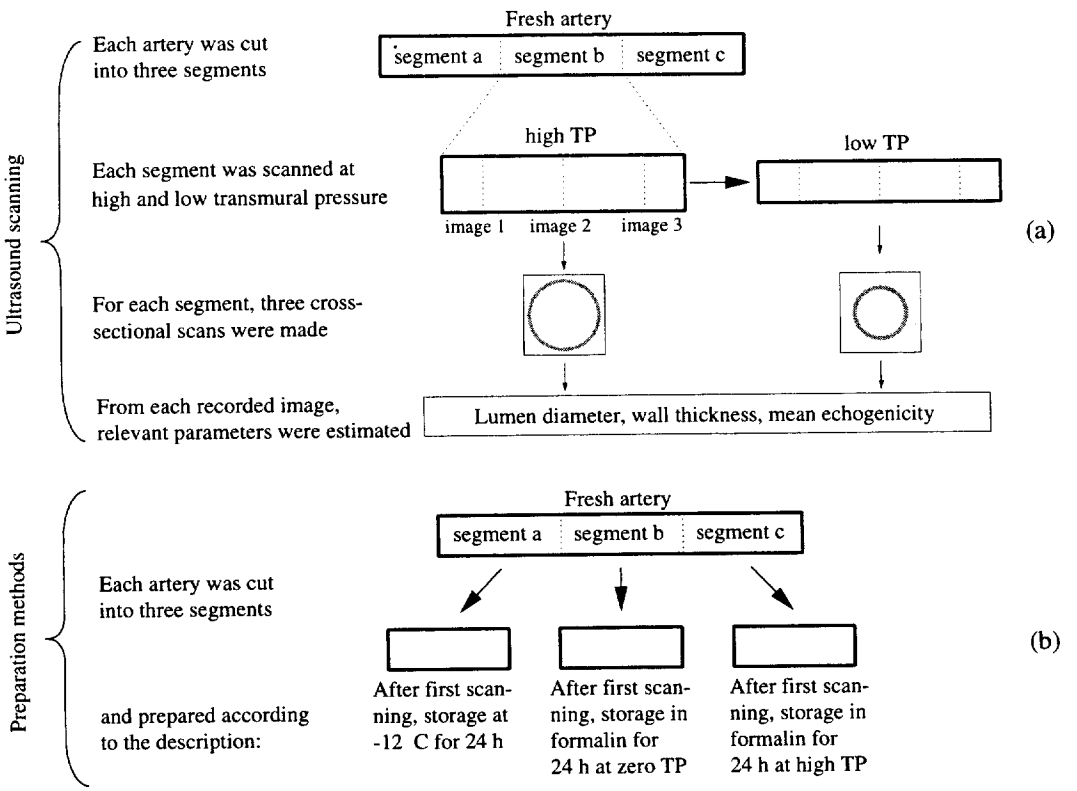

Fig. 1. Schematic illustration of artery handling and preparation methods. (a) All 5 arteries were cut into 3 segments and scanned. (b) After scanning the 3 artery segments in fresh condition, the segments were prepared. They were eventually scanned again after preparation. TP = transmural pressure.

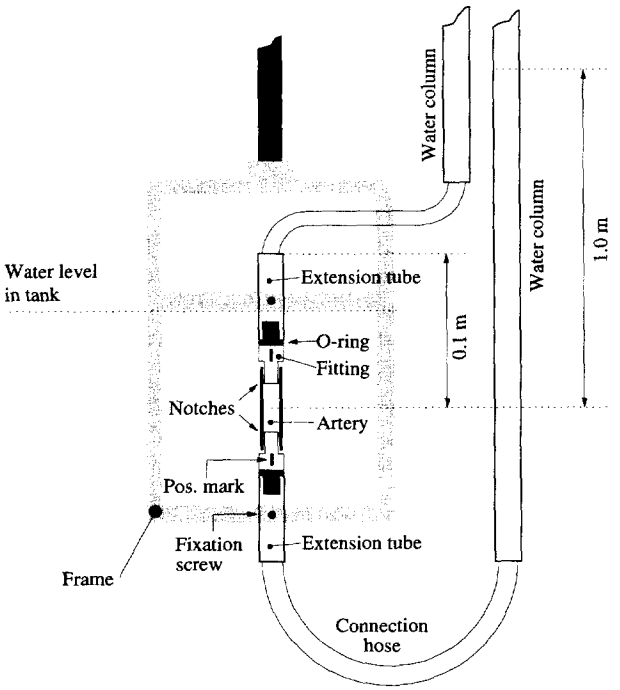

Fig. 2. Illustration of vessel holder with artery segment submerged in demineralized degassed water. The artery segment was mounted to 2 fitting assemblies, each consisting of fitting, O-ring and extension tube.

tubes was mounted onto a vessel holder, as illustrated in Fig. 2. Marks on the front of the fittings were used to ensure correct alignment. To obtain an appropriate transmural pressure, the extension tubes were connected to 2 vertically mounted tubes containing a column of demineralized degassed water. Cross-sectional ultrasound images of the artery were obtained at 2 different transmural pressures: 0.1 m and 1.0 m water column (corresponding to 7.4 mmHg and 74 mmHg, respectively). These 2 transmural pressure levels are subsequently referred to as "low" and "high." The low transmural pressure corresponded to a nearly relaxed excised artery, while the high transmural pressure roughly corresponded to a normal mean transmural pressure. For the pigs used, the mean arterial pressure (unanesthetized) was approximately in the range 80–100 mmHg.

The artery segment was always placed with its axis at the focal point of the transducer, which was located d_0 = 53 mm from the transducer's surface. This ensured roughly equal degrees of focusing on the proximal and distal wall regions. The water tank contained demineralized degassed water at 21°C with Rhodalon® (Ferrosan Fine Chemicals, Køge, Denmark) added (concentration: 1.25 % vol) to limit bacterial buildup. A saline solution, rather than demineralized water, would correspond more closely to the *in vivo* environment, but such a solution is less desirable in a scanning tank, where the sodium chloride will increase conductivity of the water and cause corrosion of metallic parts.

Measurement procedure

Cross-sectional images were recorded because lumen diameter most accurately and conveniently is identified from images in which it is possible to identify a scanline that coincides with the diameter. Specifically, lumen diameter, wall thickness and echogenicity were calculated from the echogenic image regions at the proximal and distal walls. Three cross-sectional images were obtained from each of the 15 artery segments at high and low measurement pressure, as illustrated in Fig. 1a. Images were recorded both before and after preparation. This gave a total of 180 cross-sectional images. The individual steps in the measurement process are described below.

Each of 3 fresh artery segments (from the same pig) with fittings and mounted to the extension tubes were sequentially placed in the vessel holder. The distance between the 2 rubber O-rings was measured and, by applying a tensile force along its axis, the artery was stretched longitudinally to 110% of its excised resting length to approximate typical *in vivo* length (this value was taken from previous unpublished work, where the *in vivo* length was measured before the artery was removed; information about *in vivo* length was not available in the present study). The vessel holder was next installed in the scanning tank, the extension tubes were connected to the water columns and air inside the artery segment was flushed out. The artery wall was further manipulated to remove any remaining macroscopic air bubbles attached to the vessel wall (this was always done prior to scanning). After this, 3 cross-sectional ultrasound images were recorded at low and high transmural measurement pressure. Each time the scanning was accomplished, one of the different tissue preservation methods could be commenced. After 24 h, the processed artery segments were scanned again with exactly the same spacing between O-rings as had been used in fresh state for that particular artery segment, and with the same 2 transmural measurement pressures.

Tissue preservation methods

The 3 different tissue preservation methods shown in Fig. 1b are outlined below and used on the arteries as was described above.

Freezing/thawing. Immediately after scanning (maximum 2 h postmortem), the artery segment was

detached from the vessel holder, and placed in an air-tight plastic bag (with the fittings on). This bag was placed at −12°C for 24 h. The artery segment was thawed in degassed water and subsequently reinserted into the vessel holder and scanned. Appropriate marks were used to ensure that the artery segment was reinserted at exactly the same position in the vessel holder, and with the same spacing between the O-rings.

Fixation in formalin at zero transmural pressure. The artery segment was detached from the vessel holder, and placed (with the fittings on) in degassed 5% buffered formaldehyde (formalin) at 21°C for 24 h. It was subsequently flushed with demineralized degassed water and reinserted into the vessel holder (using the same procedure as described in Freezing, above).

Fixation in formalin at high transmural pressure. The vessel holder with artery segment was removed from the scanning tank and, still under pressure, it could be inspected for possible leakages. The water inside the artery segment was removed, and the 2 extension tubes were each connected to a 2-m-long latex tube. Degassed formalin was slowly entered into one of the tubes until a formalin column of 1.0 m was reached. Care was taken to remove any air bubbles. The artery segment was left submerged in formalin for 24 h under these pressure conditions, still being longitudinally stretched. After this period of time, the artery segment was flushed with demineralized degassed water. The vessel holder (with artery) was eventually reinstalled in the scanning tank. Note that the fresh artery segment with fewest branches (of the 3 segments) was chosen for pressure fixation to minimize leakage during fixation.

Experimental ultrasound system

The experimental ultrasound system is outlined in Fig. 3. It consisted of a transducer (prototype, B&K Medical A/S, Gentofte, Denmark) connected to a diagnostic ultrasound scanner (type 3535, B&K Medical A/S) that had been modified by the company to provide the (unprocessed) radiofrequency (r.f.) signal. The transducer was a 7.5-MHz, 5-element annular array (radius of outer ring: 6.5 mm; geometric focal distance: 40 mm). Transmit focus was set electronically at d_0 = 53 mm. The received signals from the 5 rings were processed in the scanner to obtain dynamic receive focusing. Otherwise, the scanner functioned as a simple pulser/receiver. The transducer was mounted in a 3-D translation system (type 403020, Dyrbæk Technologies A/S, Åbenrå, Denmark) so that parallel B-mode images could be recorded at different axial positions of the artery (scanning of the ultrasound beam was solely done by the 3-D translation system). The r.f. signal from the scanner was digitized by a digital storage oscilloscope (DSO) (type 9450, LeCroy Corporation, Geneva, Switzerland). The DSO was connected via a GPIB interface to a Windows NT-based workstation. By use of an RS232 interface, the 3-D translation system was connected to this workstation as well. Two specially developed software pack-

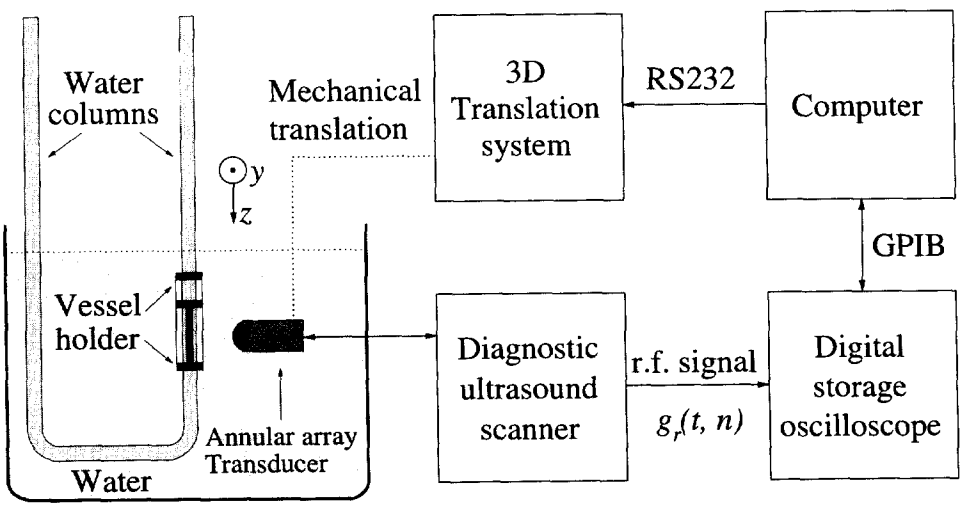

Fig. 3. Schematic illustration of the equipment used in experimental setup. $g_r(t, n)$ denotes the received r.f. signal measured in volts from scanline number n. The transducer was moved mechanically along the y-axis to record a cross-sectional image. By mechanically moving the transducer in the z-direction, 3 cross-sectional images could be recorded.

ages allowed control of the DSO and the 3-D translation system directly from the data processing and visualization program MATLAB® (The Mathworks Inc, Natick, MA).

The DSO control program allowed direct setup of all relevant instrument parameters. The program also automatically adjusted the vertical setting of the DSO (*i.e.*, voltage per division), to ensure that the dynamic range of the analog-to-digital converter was used optimally for each individual signal.

The ultrasound system was characterized with a 0.1-mm-diameter glass sphere moulded in an agar block. At the range of distances from the transducer, where the artery segment was located, the −6-dB axial resolution was approximately 0.3 mm and the −6-dB lateral resolution was 1.1 mm.

Signal processing

The signal-processing scheme is outlined in Fig. 4. The raw r.f. signals were digitized with 8 bits at a sampling rate of $f_{s,raw}$ = 400 MHz. The high sampling frequency was used to avoid aliasing of high frequency noise components into the passband of the transducer. The digitized data were transferred to the computer, where subsequent processing took place. A bandpass filter was applied to the digitized r.f., signals followed

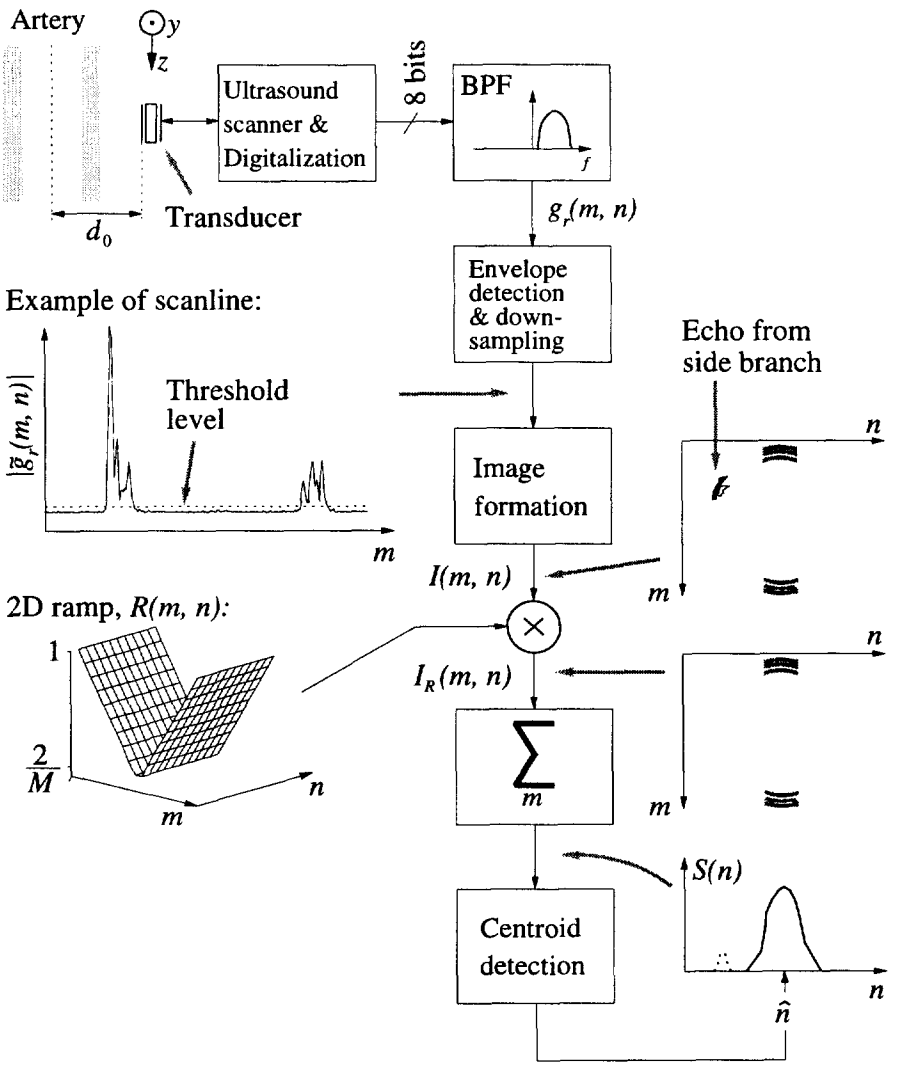

Fig. 4. Schematic illustration of signal processing for estimation of center scanline. In the coordinate system (upper left), y corresponds to the scanline number, n, and different values of z correspond to different images. $|\tilde{g}_r(m, n)|$ is the magnitude of the analytic version (denoted by $\sim$) of the received signal. The multiplication of $I(m, n)$ by $R(m, n)$ shows (in schematic form) how multiplication with the 2-D ramp is suppressing echoes from branches at the side of the artery wall. These side branches would otherwise have shown up as the dotted "artifact" in $S(n)$. BPF = bandpass filter.

by envelope detection and down-sampling (final sampling frequency, f_s = 10 MHz). Seventy-five envelope-detected signals (scanlines) placed next to each other formed one image, $I(m, n)$, where m is the row number and n is the column number [$m = 1 \ldots M$ and $n = 1 \ldots N$, where $(M, N) = (266, 75)$]. The spacing between the scanlines was 0.35 mm. The center of each artery cross-section was roughly placed in the center of the ultrasound image.

The lumen diameter and wall thickness measurements were taken at the scanline that coincided with the largest distance from the proximal to the distal wall. This "center scanline" was found automatically with the following procedure, illustrated in Fig. 4. The center scanline was estimated as the vertical line of symmetry in the image. Unfortunately, this symmetry was disturbed by possible side branches at the lateral parts of the vessel wall (*e.g.*, such as that occurring in Fig. 5, upper row, 3rd and 4th columns). To suppress such possible branches, the procedure created a new image, $I_R(m, n)$, by multiplying the image, $I(m, n)$, with a 2-dimensional ramp function with increasing values towards the proximal and distal parts of the picture and constant value perpendicular to the scanline, as illustrated in Fig. 4. From the resulting image, $I_R(m, n)$, the sum of all samples in a scanline was found:

$$S(n) = \sum_{m=1}^{M} I(m, n)R(m, n) = \sum_{m=1}^{M} I_R(m, n), \quad n = 1,2,3 \ldots N \quad (1)$$

The resulting projection, $S(n)$, is sketched in Fig. 4 as well. From the centroid ("point of gravity") of this projection, the scanline coinciding with the diameter could be estimated;

$$\hat{n} = round\left(\frac{\sum_{n=1}^{N} n\,S^2(n)}{\sum_{n=1}^{N} S^2(n)}\right) \quad (2)$$

However, in 9 of the 180 images, the disturbances from branches at the sides of the vessel were so severe (as revealed by manual inspection of the estimated scanline superimposed on the ultrasound image) that manual detection of the scanline had to be used.

A peak detection algorithm was next applied to

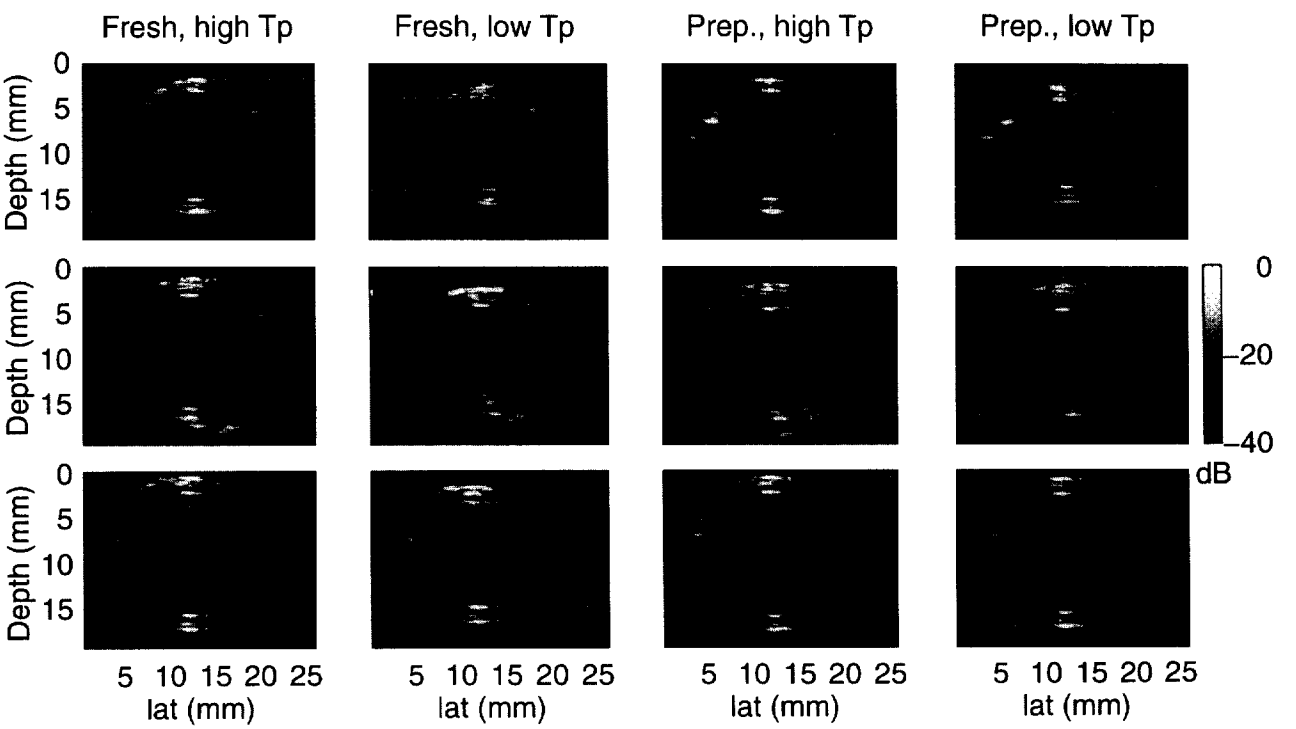

Fig. 5. Logarithmic compressed, cross-sectional images of the same porcine artery segment recorded fresh and prepared, with low and high transmural measurement pressure. The 3 rows correspond to: freezing/thawing, fixation in formalin at zero transmural pressure, and fixation in formalin at high transmural pressure. Note the side branches at the distal wall in the second row of images.

the center scanline to estimate the location of the 4 peaks that corresponded to the following 4 interfaces: 1. Water to outer wall; 2. inner wall to water in lumen; 3. water in lumen to inner wall; and 4. outer wall to water. Detection of the correct peak from the envelope signal was sometimes difficult because the received signal contained noise and the peak in question was disturbed by interfering components due to layers in the wall close to the surface and due to the vessel wall not being a smooth surface (*e.g.*, fragments of connective tissue can appear on the outer wall surface). To help identify the correct peak, a threshold (depicted in Fig. 4 as well) was used with the peak detection procedure, so that only peaks with values above the threshold value were considered representative of the interfaces in question. The threshold value was set at a value slightly larger than the noise level, and iteratively adjusted while visually inspecting the envelope of center scanlines with the peaks superimposed.

From the location of the peaks, the inner diameter and the wall thickness could be estimated. Only the proximal wall was used for the thickness calculation because this wall was much better defined than the distal wall. Specifically, due to the relatively large change in acoustic impedance between water and wall material, there appeared to be quite high transmission losses through the proximal wall, with respect to the echo signal received from the distal wall. This resulted in a reduced signal-to-noise ratio (SNR) for the signal from the distal wall, compared to the SNR in the signal from the proximal wall. All calculations were based on a sound speed of 1480 m/s.

An estimate of the mean echogenicity of the wall

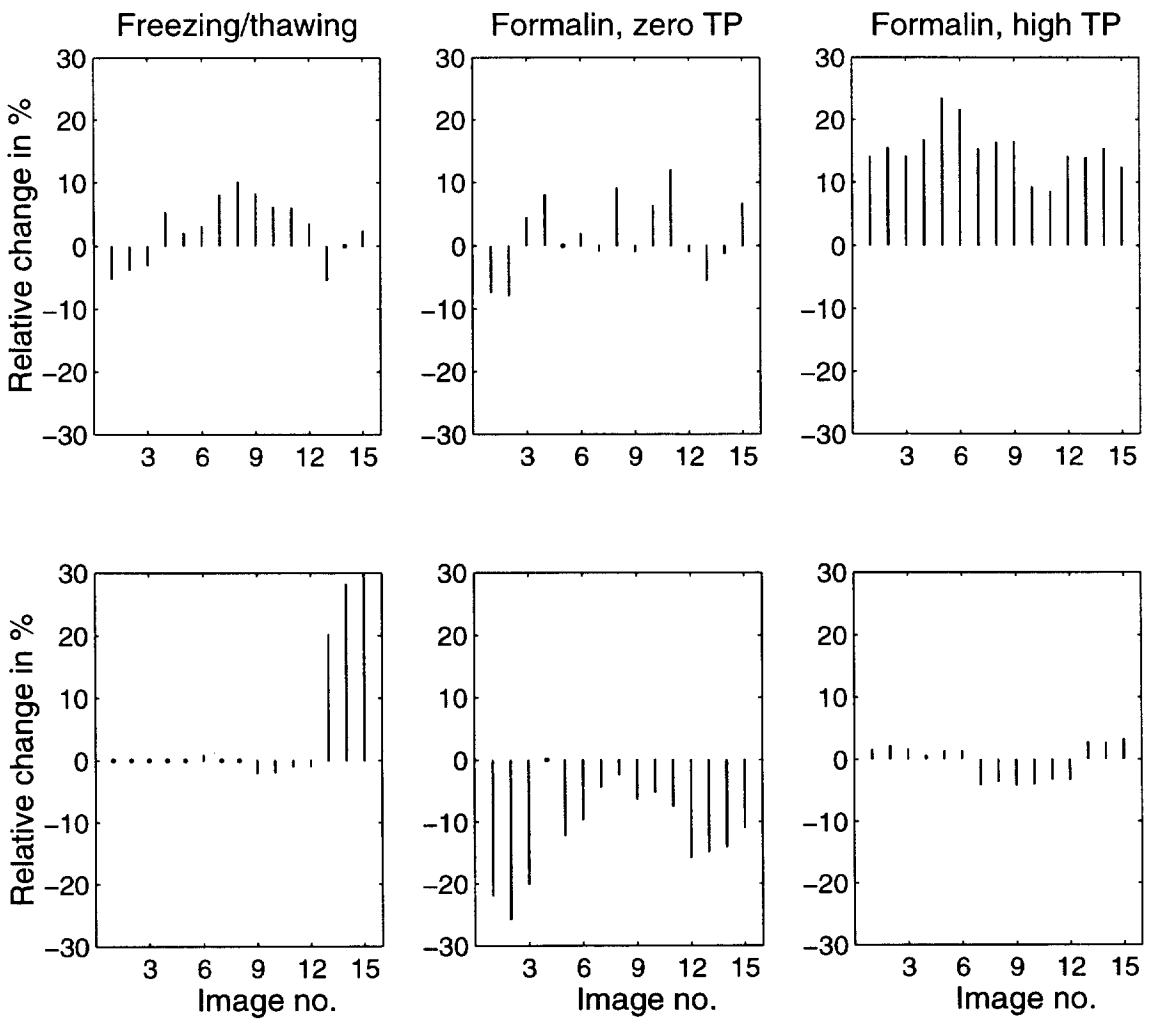

Fig. 6. Relative lumen diameter changes in percent. The 3 columns correspond to the 3 different preparations. The upper and lower rows show the relative change (from prior to preparation to after preparation) found when measuring at low and high transmural pressure, respectively.

was calculated in the following manner. A region containing 5 scanlines, located around the center scanline, was isolated at the proximal and distal walls. Because the echoes from the 4 interfaces between artery wall and water are irrelevant for the echogenicity of the artery wall itself, the parts in the received signal that originated from these interfaces (temporal range is approximately half a −6-dB pulse length from the peak) were excluded. The duration of the remaining signal segment was denoted T_{gate}, and the proximal or distal wall signals could then be extracted with a rectangular window, $g_{gate}(m)$, of width $M_{gate} = T_{gate}f_s$. The *rms* value of the proximal and distal wall regions was then calculated as:

$$\hat{g}_{rms} = \sqrt{\frac{1}{5\,M_{gate}} \sum_{n=\hat{n}-2}^{\hat{n}+2} \sum_{m=-\infty}^{\infty} |g_r(m, n) g_{gate}(m)|^2} \quad (3)$$

where $\hat{n}$ is the index of the center scanline for the given artery segment found in eqn (2) and $g_r(m, n)$ is the discrete received signal associated with the nth scanline.

Statistics

The p values identified in Results were calculated with Wilcoxon matched-pairs signed-ranks test. The null hypothesis is that the difference ($d_i = x_i - y_i$) between the members of each pair (x_i, y_i) has median value zero. The pairing of the data was done in such a way that the condition for pressurization was the same before and after preparation. Changes due to fixation are given as mean ± standard deviation, followed by the p value. The number of observations was always 15.

RESULTS

Typical examples of recorded images are shown in Fig. 5. The specular reflection from wall structures

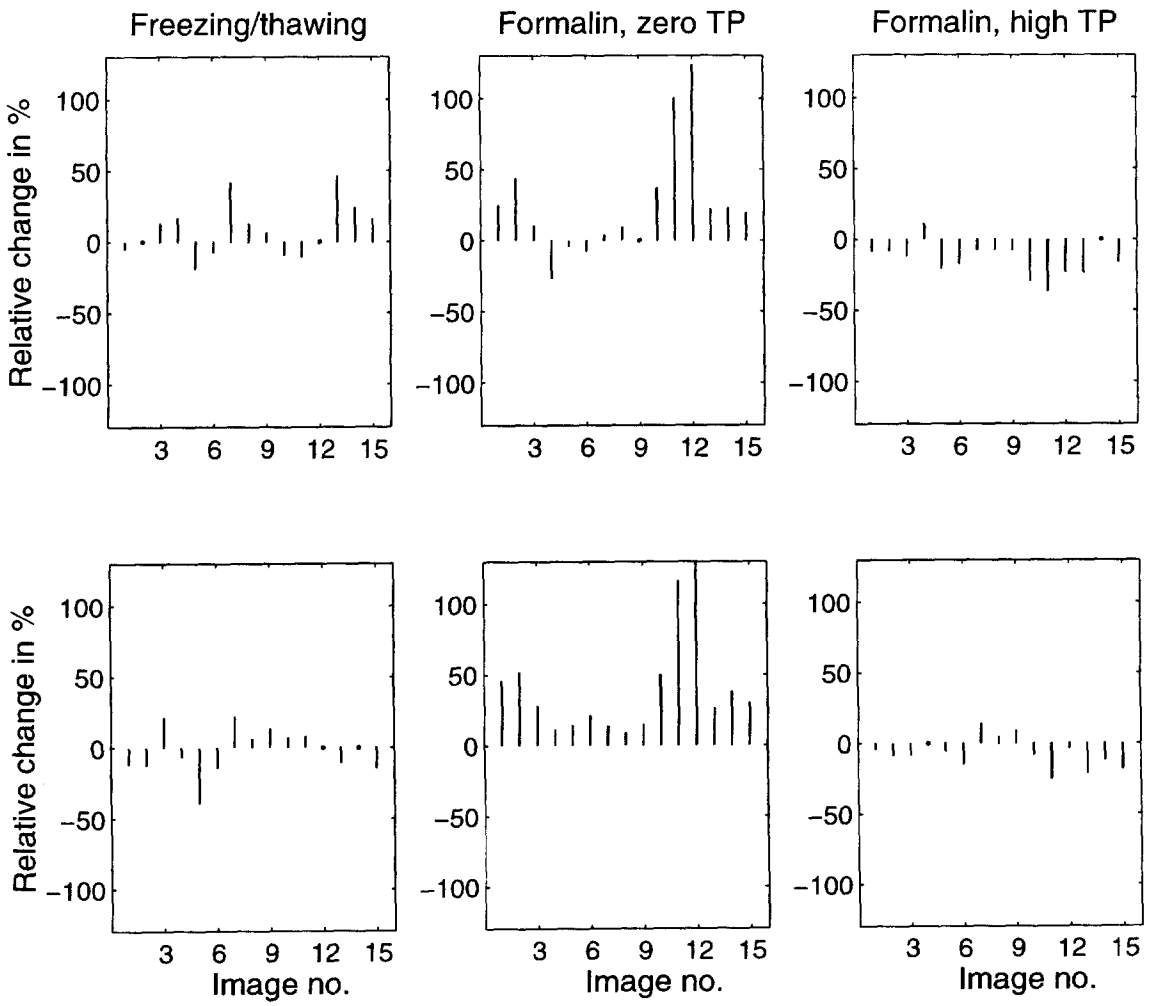

Fig. 7. Relative wall thickness changes in percent for the 3 groups of preparations. The layout is identical to that of Fig. 6.

Table 1. Summary of relative changes due to fixation in lumen diameter, wall thickness and echogenicity.

Item	TP (mmHg)	Freezing/thawing ($\mu \pm \sigma\%$ p)	Formalin, zero TP ($\mu \pm \sigma\%$ p)	Formalin, high TP ($\mu \pm \sigma\%$ p)
Lumen diameter	7.4	3 ± 5% NS	2 ± 6% NS	15 ± 4% 0.0007
	74	0 ± 1% †NS	−11 ± 7% 0.001	0 ± 3% NS
Wall thickness	7.4	8 ± 19% NS	25 ± 40% 0.02	−14 ± 12% 0.002
	74	−1 ± 17% NS	40 ± 37% 0.0007	−7 ± 11% NS
Echogenicity	7.4	28 ± 43% 0.02	−21 ± 38% 0.01	−1 ± 36% NS
	74	14 ± 52% NS	−25 ± 37% 0.01	−13 ± 29% 0.05

Figures are given as mean (μ) ± standard deviation (σ) in percent, followed by the p value (n = 15). A p value larger than 0.05 is indicated by NS (not significant). TP = transmural pressure; 7.4 mmHg and 74 mmHg = "low" and "high" transmural pressure, respectively.

† The last 3 measured values were discarded, because the measurement pressure was erroneously set to "low" during recording, when it should have been set to "high".

perpendicular to the ultrasound beam (*e.g.*, top, bottom and possible side branches) is noted. On each image, a vertical dotted line indicates the automatically detected center scanline and 4 horizontal dotted lines indicate the four previously described peaks designating the 4 water/wall interfaces. The locations of the latter 4 lines were used in the calculations of lumen diameter and vessel wall thickness.

The lumen diameter of the fresh relaxed arteries ranged from approximately 6 mm to 12 mm and the wall thickness from approximately 1 mm to 2 mm. The uncertainty on a single peak location estimate was estimated to be approximately one wavelength or 0.2 mm.

Lumen diameter and wall thickness

From the location of the peaks at the center scanline, the lumen diameter and the wall thickness were found. The results of calculating the relative change in lumen diameter and wall thickness, due to a given tissue preservation method, are given in Figs. 6 and 7 and summarized in Table 1. Each plot contains a total of 15 bars, 3 for each of the 5 artery segments.

In the low transmural-measurement-pressure group (Fig. 6, upper row), no significant changes were observed for the artery segments that had been frozen and for those that had been prepared in formalin at zero transmural pressure. For the arteries fixed in formalin at high transmural pressure, a 15 ± 4% ($p < 0.0007$) increase in lumen diameter was observed (*i.e.*, the artery fixed under transmural pressure did not contract when the transmural measurement pressure was lowered).

Turning to the high transmural-measurement-pressure group (Fig. 6, lower row), no changes were observed for the artery segments that had been frozen, except for the fifth artery segment, which showed a strong increase in lumen diameter. The latter observation was most likely due to an erroneous constant low transmural-measurement-pressure setting during scanning in fresh state (from the recorded images in fresh state, the diameter of the artery segment was found to be the same at low and high transmural-measurement pressure). For the arteries fixed in formalin at zero transmural pressure, significant decreases in lumen diameter ($-11 \pm 7\%$, $p < 0.001$) were observed (*i.e.*, the artery fixed in formalin at zero transmural pressure did not expand when high transmural-measurement pressure was applied). For the arteries fixed in formalin at high transmural pressure, small random changes were observed, which is in accordance with the observed increase in lumen diameter at low transmural-measurement pressure.

The results of the wall thickness changes are shown in Fig. 7. In the low transmural-measurement-pressure group (Fig. 7, upper row), no significant changes were observed for the artery segments that had been frozen. For the arteries fixed in formalin at zero transmural pressure and at high transmural pressure, mainly increases ($25 \pm 40\%$, $p < 0.02$) and decreases ($-14 \pm 12\%$, $p < 0.002$) in wall thickness were observed, respectively.

In the high transmural-measurement-pressure group (Fig. 7, lower row), only random changes were observed for the frozen artery segments. For the arteries fixed in formalin at zero transmural pressure, large positive changes ($40 \pm 37\%$, $p < 0.0007$) were found, which is in accordance with the results for the lumen diameter. Eventually, for the arteries fixed in formalin at high transmural pressure, the changes were random and relatively small, again indicating that the prepared artery had the same dimensions as the fresh artery, at high transmural-measurement pressure.

Mean echogenicity

Estimates of the mean echogenicity were found for the proximal and distal wall regions. The level of echogenicity was higher for the proximal wall than the distal wall. This difference was mainly due to the transmission

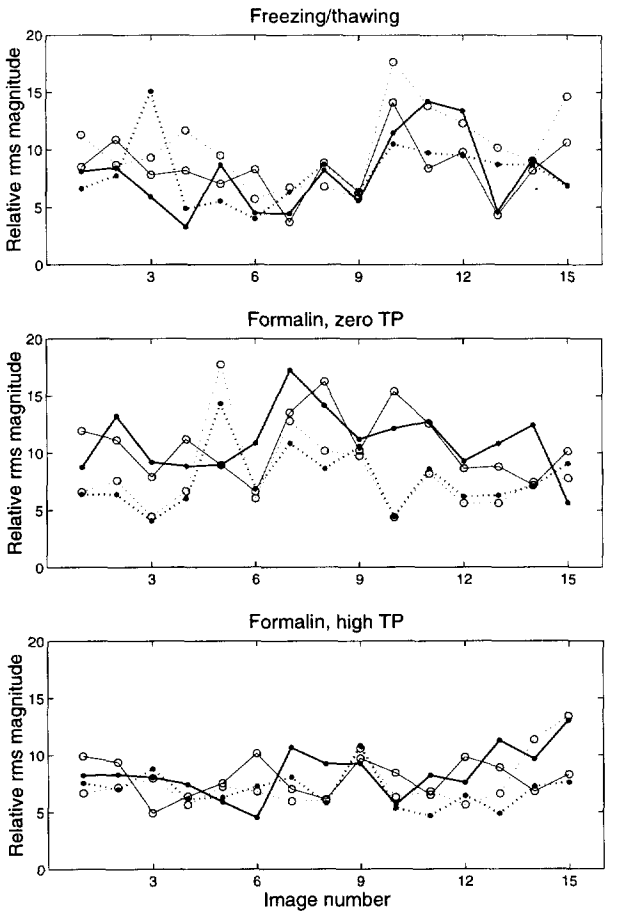

Fig. 8. Estimate of mean echogenicity (*rms* value of received signals in wall region) for proximal wall. (—○—) and (—●—) indicate fresh arteries under low and high transmural measurement pressure, respectively. (. . . ○ . . .) and (. . . ● . . .) indicate prepared artery segments under low and high transmural measurement pressure, respectively. The data points, which represent independent estimates of echogenicity, are connected only to ease interpretation. The vertical scale is calibrated in the same arbitrary units for all 3 plots.

loss at the proximal wall, for the echo signal originating from the distal wall. Thus, only echogenicity of the proximal wall is of interest, and the levels are shown in Fig. 8 and summarized in Table 1.

For fresh artery segments, echogenicities at low and high transmural measurement pressure were compared via a student's *t*-test. No statistical significance was found due to change in transmural measurement pressure.

The variation in echogenicity was rather large, as can be observed from Fig. 8. Comparing the levels before and after preparations reveals that only for artery segments fixed in formalin at zero transmural pressure, a consistent (*i.e.*, both at low and high transmural measurement pressure) reduction ($-23 \pm 38\%$, $p < 0.01$) in echogenicity was found (dotted lines appear below solid lines).

In general, for the 2 tissue preservation methods involving formalin, the levels of echogenicity of the prepared artery were approximately independent of transmural measurement pressure.

DISCUSSION

The dimension of the fresh (elastic) artery was basically determined by the transmural (measurement) pressure: when the transmural pressure was raised, the artery distended, resulting in a thinner wall. This effect was present in all fresh arteries. With this in mind, the results for the geometrical distortion observed can be interpreted as follows.

Freezing/thawing. Although the changes in lumen diameter and wall thickness fluctuated at low transmural-measurement pressure, the changes in these 2 measures became smaller at high transmural-measurement pressure. Specifically, at high transmural-measurement pressure, the lumen diameter of the first 4 artery segments stayed remarkably constant. One reason for the large fluctuation at low transmural-measurement pressure can be that the "local" geometry of the artery is more influenced by shear forces (due to nonstraight artery, ligations, sutures, branches, etc.) compared to the high transmural-measurement-pressure situation. In conclusion, there was no indication of a consistent geometrical distortion due to freezing/thawing.

Fixation in formalin at zero transmural pressure. The changes in lumen diameter due to fixation were not significant when evaluated at low transmural-measurement pressure but, at high transmural measurement pressure, a consistent reduction of 11% was seen. The wall became more rigid. Loss of distensibility has also been documented by Park et al. (1993) when examining healthy human arteries before and after fixation. The loss of elasticity was also supported by the, on average, 40% greater vessel wall thickness found for fixed arteries compared with fresh when measuring at high transmural pressure (the fresh vessel wall became thinner when the high transmural-measurement pressure increased the lumen area, whereas this effect failed to appear in the prepared artery). The 40% increase was not only due to loss of distensibility because, at low transmural measurement pressure, there was a general tendency toward an increase in wall thickness due to fixation, indicating a swelling of the arterial wall tissue. This may be similar to the swelling observed following formalin fixation of mildly arteriosclerotic arteries (Siegel et al., 1985) or other tissues (Bamber et al., 1979). If the corresponding images

are examined, a quite peculiar-looking artery cross-section was frequently observed, indicating uneven swelling of wall tissue (which might be due to a non-straight artery, ligations, sutures, branches, etc.). This type of observation was especially pronounced for the 2 very large increases in wall thickness in Fig. 7 (second column). This effect also disturbed the peak detection algorithm.

Fixation in formalin at high transmural pressure. The finding of an average increase in lumen diameter of 15% at low transmural-measurement pressure, and no consistent effect at high transmural-measurement pressure, indicates that the prepared artery maintained its dimensions (the dimension of the prepared artery was independent of transmural-measurement pressure). The same conclusions can be found from the changes in wall thickness, even though the standard deviation was larger here (Table 1).

The results for the average *rms* value of the echo signal, $\hat{g}_{\text{rms}}$, from the inside of the proximal wall, only changed significantly (at both low and high transmural measurement pressure) for the segments fixed in formalin at zero transmural pressure. The decrease was approximately 23% ($p < 0.01$). Due to the swelling of the wall of these arteries, the wall might then contain more water (or formalin), yielding a decrease in echogenicity. The large standard deviation on $\hat{g}_{\text{rms}}$ was due to: 1. Strong interference between the received wave fronts from the different closely spaced layers of the artery wall; and 2. the relatively short observation window, T_{gate}. For these reasons, the results for echogenicity should be confirmed by a larger study.

With respect to the frozen/thawed arteries, a number of research groups have applied slightly different techniques for measuring other acoustic properties (*e.g.*, attenuation, backscattering) for other types of tissues. To our knowledge, no significant changes have been reported due to freezing/thawing (Crosby & Mackay 1978; van der Steen et al. 1991; Parker 1983).

Figure 8 also reveals that the mean echogenicity of the fresh arterial wall, for the segments that were to be fixed in formalin at zero transmural pressure, unexpectedly appeared somewhat higher (on average 20%) compared to the mean echogenicity of all fresh arteries ($p < 0.001$, calculated with a student's *t*-test). The artery segments were not always prepared in the same order as they appeared *in vivo* because fixation in formalin at high transmural pressure was done on the artery segment with the least number of branches, as mentioned previously. On the other hand, the total length of fresh arteries (before cutting into 3 segments) was approximately 90 mm, and the histological composition is, thus, unlikely to be very different among the 3 artery segments. Nevertheless, there is a possibility that the observed higher level of echogenicity for the arteries that were to be fixed with formalin at zero transmural pressure could be attributed to variation in arterial properties.

A potential problem in ultrasonic *in vitro* measurement of echogenicity is the presence of air in the tissue specimen (*e.g.*, in connection with autolysis). In the present study, the tissue specimens were not degassed in vacuum nor was the solubility of air in the tissue increased by pressurization nor by storage at low temperature (Bamber & Nassiri 1985). Because none of the levels of echogenicity are dramatically different from the mean, there was a strong indication that air was not present in the artery segments investi-

Table 2. Overview of advantages and disadvantages of the condition of arteries after processing with the 3 different tissue preservation methods.

Method	Advantages	Disadvantages
Freezing at −12°C for 24 h with subsequent thawing	Behaves roughly as freshly excised artery	Continues to degrade while not in frozen condition
Fixation in formalin at zero transmural pressure at 21°C for 24 h	Probably no degradation during subsequent submersion in water for a short period of time	Physically fixed in a deformed shape relative to fresh artery Does not respond to change in transmural pressure Decrease in echogenicity
Fixation in formalin at 21°C for 24 h with 74 mmHg of transmural pressure and longitudinal stretching by tensile force	Probably no degradation during subsequent submersion in water for a short period of time Physically fixed in a shape that corresponds to the shape of the fresh artery at the given transmural pressure	Does not respond to change in transmural pressure

"Water" is assumed to be demineralized degassed water.

gated. This seems consistent with the fact that the arteries were kept in degassed water and prepared very quickly *post mortem*. On the other hand, the moderate increase in echogenicity due to freezing/thawing (Table 1) could, in principle, be associated with air bubbles.

As a guide in selecting the proper tissue preservation method for a given *in vitro* study, Table 2 lists advantages and disadvantages of the 3 methods, as revealed by the present investigation.

CONCLUSIONS

The present study demonstrated that arterial tissues react differently to different methods of preservation. Fixation in formalin at zero transmural pressure caused significant swelling ($25 \pm 40\%$, $p < 0.02$) and a decrease ($-23 \pm 38\%$, $p < 0.01$) in echogenicity of the arterial wall. Such changes could not be found when the arteries underwent freezing and subsequent thawing nor when the arteries were fixed with formalin at 74 mmHg transmural pressure and stretched. Thus, the latter 2 methods are more appropriate procedures of fixation when conducting *in vitro* studies of arterial diseases, such as wall thickening due to arteriosclerosis.

Due to the difference in functionality of the frozen/thawed artery and the artery fixed under pressure, only the first method can be used prior to experiments that aim toward simulating an artery in the relaxed state. However, if an artery is to be simulated during systole, both methods can be used.

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